

Single-cell whole-genome amplification technique impacts the accuracy of SNP microarray-based genotyping and copy number analyses

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ABSTRACT: Methods of comprehensive microarray-based aneuploidy screening in single cells are rapidly emerging. Whole-genome amplification (WGA) remains a critical component for these methods to be successful. A number of commercially available WGA kits have been independently utilized in previous single-cell microarray studies. However, direct comparison of their performance on single cells has not been conducted. The present study demonstrates that among previously published methods, a single-cell GenomePlex WGA protocol provides the best combination of speed and accuracy for single nucleotide polymorphism microarray-based copy number (CN) analysis when compared with a REPLI-g- or GenomiPhi-based protocol. Alternatively, for applications that do not have constraints on turnaround time and that are directed at accurate genotyping rather than CN assignments, a REPLI-g-based protocol may provide the best solution.

Key words: whole-genome amplification / SNP microarray / copy number / single-cell genotyping / aneuploidy screening

Introduction

Many groups have developed whole-genome microarray-based methods to assess chromosome copy number (CN) in order to diagnose aneuploidy in human embryos from a single cell (Handyside *et al.*, 2009; Vanneste *et al.*, 2009; Gutierrez-Mateo *et al.*, 2010; Johnson *et al.*, 2010a; Treff *et al.*, 2010a). These developments are in large part due to the failure of fluorescence *in situ* hybridization (FISH)-based methods to result in the expected clinical benefit of aneuploidy screening for the treatment of infertility (reviewed in Fritz, 2008). Genome-wide approaches are certainly more comprehensive than FISH (24 compared with ≤ 12 chromosomes, respectively) and some microarray-based methods have shown significantly improved consistency (Treff *et al.*, 2010a,b) and predictive value for aneuploidy diagnosis (Scott *et al.*, 2008; Northrop *et al.*, 2010).

Some methods of 24 chromosome CN have also demonstrated accuracy of blinded predictions in single cells from a variety of cell lines with previously well-characterized karyotypic abnormalities (i.e. Treff *et al.*, 2010a). Unfortunately, other studies have considered a method to be accurate by only establishing that two different methods of analysis indicate that an embryo is abnormal even if the results of the two tests

indicate that the abnormalities involved completely different chromosomes (Gutierrez-Mateo *et al.*, 2010). This may be inadequate to establish the accuracy of a test for single-cell 24 chromosome aneuploidy diagnosis. Even more troubling is the lack of any accurate calculations after analysis of single cells from cell lines with known abnormalities by technologies such as comparative genomic hybridization (CGH) or array-CGH. Some microarray-based studies have performed testing of single cells from cell lines (Vanneste *et al.*, 2009; Johnson *et al.*, 2010a). However, one study suggested that the method was accurate after evaluating only a small number of single cells ($n = 7$) from cell lines, was unable to obtain an interpretable result in 41% of blastomeres evaluated, and required analysis by two arrays; bacterial artificial chromosome (BAC) and single nucleotide polymorphism (SNP) (Vanneste *et al.*, 2009). This may not represent sufficient validation, reliability or feasibility for routine clinical application. Although the second study involving cell lines (Johnson *et al.*, 2010a) did evaluate a large sample size ($n = 459$), only a single type of abnormality (trisomy 21) was represented. This may also be inadequate to determine the accuracy of predicting aneuploidy for all 24 chromosomes.

Unfortunately, these and other preclinical validation considerations have gone overlooked during the development and implementation of

many new technologies for 24 chromosome aneuploidy screening. Clinical studies have also been limited. For example, case–control and observational studies may not represent sufficient strength of evidence to determine the clinical validity of new technologies. This is particularly true in light of the experiences with FISH-based aneuploidy screening which was suggested to be clinically beneficial based on case–control studies (Gianaroli et al., 1997; Munne et al., 1999; Kahraman et al., 2000; Munne et al., 2003; Munne et al., 2006), despite failing to show a meaningful benefit in all randomized controlled clinical trials (reviewed in Fritz, 2008). Although similar case–control and observational clinical studies using comprehensive methods of aneuploidy screening have been reported (Wells et al., 2009; Munne et al., 2010; Rabinowitz et al., 2010; Schoolcraft et al., 2010a,b), randomized controlled trials have been limited. Indeed, class I strength of evidence for a significant improvement in clinical pregnancy and embryo implantation rates (i.e. Scott et al., 2010) should be made standard for any new aneuploidy screening technology prior to routine implementation. An equally important clinical trial involves a prospective blinded non-selection design (i.e. Scott et al., 2008) in which the negative predictive value of the test is determined. In other words, it is critical to know whether the test produces false-positive abnormal diagnoses in embryos that are otherwise capable of developing into chromosomally normal pregnancies. Such a study is important in confirming whether the test can be used to safely discard human embryos.

Whole-genome amplification

A critical step in every single-cell 24 chromosome aneuploidy screening method is whole-genome amplification (WGA). Single cells possess ~6–7 pg of genomic DNA (gDNA) (Dolezel et al., 2003) and microarrays typically require nanogram amounts of DNA to proceed as recommended. This necessitates amplification of the genome by more than 1000-fold. Moreover, since these technologies have commonly been applied to quantitatively evaluate chromosomal CN, the WGA procedure must result in unbiased amplification to maintain relative quantities of DNA across the entire genome. Some methods of WGA and microarray-based molecular karyotyping rely upon the interpretation of qualitative genotypes rather than quantitative CN assignments (Handyside et al., 2009; Johnson et al., 2010a). In these situations and in applications where single gene disorders may be evaluated directly (Hellani, 2005; Burlet et al., 2006; Lledo et al., 2006; Panelli et al., 2006; Lledo et al., 2007; Ren et al., 2007; Renwick et al., 2007; Hellani et al., 2008a) or through microarray-based haplotype inheritance analyses (Handyside et al., 2009), genotyping fidelity is also a critical component of WGA.

There are a variety of commercially available reagents to perform single-cell WGA that have aided in widespread utilization (Table I). For example, some groups have used a multiple displacement amplification (MDA) approach using QIAgen's 'REPLI-g' technology (Handyside et al., 2004, 2009; Sher et al., 2007, 2009) or GE Healthcare's 'GenomiPhi' technology (Le Caignec et al., 2006; Hellani et al., 2008b; Vanneste et al., 2009). MDA involves the use of a bacteriophage (Φ 29) DNA polymerase that employs rolling circle amplification during incubation at a single temperature (isothermal) (Dean et al., 2002). Other groups have employed PCR-based amplification strategies using Sigma's 'GenomePlex' technology (Fiegler et al., 2007;

Gutierrez-Mateo et al., 2010; Treff et al., 2010a). PCR-based WGA involves the use of a DNA polymerase from the thermophilic bacterium *Thermus aquaticus* and repeated cycling between temperatures appropriate to sequentially denature and elongate the DNA (Saiki et al., 1988). Interestingly, comparison studies of commercially available MDA and PCR-based WGA methods have only evaluated the performance on input DNA quantities that exceed those found in a single cell (Lovmar et al., 2003; Barker et al., 2004; Park et al., 2005). The present study performs the first direct comparison of commercially available single-cell WGA methodologies for amplification reliability, fidelity and accuracy by SNP microarray analysis.

Materials and Methods

Experimental design

This study was designed to evaluate three commercially available methods of WGA on single cells. The evaluation was conducted using an SNP microarray platform with gDNA extracted from a large amount of cells serving as a benchmark for genotyping and CN accuracy on single cells from the same cell line.

Single-cell isolation

Four human fibroblast cell lines were obtained from the Coriell Cell Repository (Camden, NJ, USA). The karyotype of each cell line was different in the CN of the X chromosome and included a 46,XY cell line (GM00323) representing a chromosome X CN of 1, a 46,XX cell line (GM00321) representing a chromosome X CN of 2, a 47,XXX cell line (GM04626) representing a chromosome X CN of 3 and a 49,XXXXY cell line (GM00326) representing a chromosome X CN of 4. Cells were cultured in Eagle's minimum essential medium with 15% fetal bovine serum, 2× non-essential amino acid and 1% penicillin–streptomycin–glutamine (Invitrogen Corp., Carlsbad, CA, USA) at 37°C and 5% CO₂. Single cells were isolated following treatment with trypsin/EDTA (Invitrogen) to detach the adherent fibroblast cultures as recommended. Single cells were then picked up in 1 µl of media using a 100 µm stripper tip (Midatlantic Diagnostics, Mount Laurel, NJ, USA) under a dissecting microscope and placed in the bottom of a 0.2 ml PCR tube (Ambion Inc., Austin, TX, USA) holding WGA method-specific solutions as described below. Thirty single cells were picked up from each cell line; 10 single cells for each WGA method. One microlitre of media was removed to serve as negative controls for each WGA method. gDNA was also extracted from each cell line immediately after single cells were obtained using the DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA, USA) as described by the manufacturer.

Single-cell WGA

The GenomiPhi DNA amplification kit (GE Healthcare, Piscataway, NJ, USA) was used on single cells according to a previous publication (Le Caignec et al., 2006). One microlitre of culture media containing a single cell was loaded into 0.2 ml PCR tubes containing 2.5 µl alkaline lysis buffer [200 mM KOH and 50 mM DTT (Cui et al., 1989)]. The samples were stored at –80°C for at least 30 min and then incubated at 65°C for 10 min. Two and a half microlitres of neutralization buffer [0.9 M Tris–HCl, pH 8.3, 0.3 M KCl and 0.2 M HCl (Cui et al., 1989)] were then added to the sample to neutralize the lysis buffer. Nine microlitres of GenomiPhi sample buffer containing the random hexamer primers were added to the neutralized cell lysate, followed by 9 µl of GenomiPhi reaction buffer and 1 µl of GenomiPhi enzyme mix. The isothermal amplification was performed at 30°C for 3 h and the reaction was stopped upon incubation at 65°C for 10 min.

Table I Comparison of notable 24 chromosome aneuploidy screening technologies.

Characteristic	CGH		SNP array			Array-CGH		SNP array + array-CGH
	Wells et al. (1999)	Sher et al. (2007)	Johnson et al. (2010a)	Treff et al. (2010a)	Handyside et al. (2009)	Gutierrez-Mateo et al. (2010)	Hellani et al. (2008b)	Vanneste et al. (2009)
WGA method	PCR (custom DOP-PCR)	MDA (REPLI-g)	MDA (undisclosed)	PCR (GenomePlex)	MDA (REPLI-g)	PCR (GenomePlex/Sureplex)	MDA (GenomiPhi)	MDA (GenomiPhi)
Array method	NA	NA	370K SNP	250K SNP	370K SNP	2K CGH (BAC)	44K oligonucleotide	4K CGH (BAC) and 250K SNP
2-day turnaround time	—	—	+	Treff et al. (2009a)	—	+	—	—
Cell line study ^a	—	—	+	+	—	—	—	+
Consistency study ^b	Wells and Delhanty (2000)	—	+	+	—	—	—	+
FISH comparison study	Fragouli et al. (2008)	Keskintepe et al. (2007)	—	Treff et al. (2010b), Northrop et al. (2010)	—	+	+	—
Single gene disorder detection ^c	—	—	Rabinowitz et al. (2009)	Treff et al. (2009b)	Handyside et al. (2010)	—	—	—
Chromosome translocation detection	—	—	Johnson et al. (2010b)	Treff et al. (2010c)	—	Escudero et al. (2010)	—	—
Observational or case–control study	Schoolcraft et al. (2010a)	+, Sher et al. (2009)	Rabinowitz et al. (2010)	Schoolcraft et al. (2010b)	—	Munne et al. (2010)	—	—
Non-selection study	—	—	—	Scott et al. (2008)	—	—	—	—
Randomized controlled study	—	—	—	Scott et al. (2010) ^d	—	—	—	—
Deliveries reported	Wells et al. (2009)	Sher et al. (2009)	—	Treff et al. (2009a)	—	+	—	—

A '+' symbol refers to the reference cited in the header of each respective column.

^aAnalysis of accuracy on single cells with known karyotypes.

^bAnalysis of multiple blastomeres from within the same embryos.

^cDemonstrated ability to evaluate a monogenic disorder from the same biopsy.

^dThis study included demonstrating equivalence of a real-time PCR protocol (Treff et al., 2009c) to the SNP microarray protocol (Treff et al., 2010a) prior to using it in a randomized controlled trial.

The REPLI-g Midi Kit (Qiagen) was used on single cells according to a previous publication (Handyside et al., 2004). Single cells in 1 μ l of culture media were loaded into 0.2 ml PCR tubes containing 2.5 μ l PBS buffer. Three and a half microlitres of buffer D2 were added followed by a 10 min incubation on ice and a 5 min incubation at 65°C. Three and a half microlitres of stop solution were added to stop the lysis reaction. A WGA master mix containing 10 μ l nuclease free water, 29 μ l reaction buffer and 1 μ l DNA polymerase was added to the cell lysate followed by the isothermal amplification at 30°C for 16 h and inactivation at 65°C for 3 min.

The GenomPlex Single Cell Whole Genome Amplification Kit (WGA4; Sigma Aldrich, St Louis, MO, USA) was used on single cells as described in a previous publication (Fiegler et al., 2007). Single cells in 1 μ l of culture media were loaded into 0.2 ml PCR tubes containing 7 μ l of nuclease free water. One microlitre of alkaline lysis buffer was added followed by incubation at 65°C for 10 min to lyse the cell. One microlitre of neutralization buffer was added to neutralize the lysis buffer. WGA was performed following the manufacturer's instructions (Sigma Aldrich).

WGA DNA from each of the three methods described above was purified using the GenElute PCR Cleanup Kit (Sigma Aldrich) as described in the manufacturer's instructions.

Single-cell WGA reliability

The concentration of purified WGA DNA and gDNA was measured using a NanoDrop 8000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and DNA yield was calculated. One hundred nanograms of WGA DNA and gDNA were loaded to 2% E-Gel electrophoresis system (Invitrogen) and visualized with a Kodak Gel Logic 100 system (Kodak, Rochester, NY, USA). Successful WGA was defined as a single-cell sample that yielded more than the required input WGA DNA amount for SNP microarray-based analysis (250 ng). For each method, reliability was defined as the percentage of samples that met this definition.

Single-cell WGA genotyping fidelity

Three representative WGA DNA samples from each WGA method and each cell line were evaluated by SNP microarray analysis. Two hundred and fifty nanograms of WGA DNA or gDNA were processed with the GeneChip 250K Nspl SNP microarray as instructed by the supplier (Affymetrix, Santa Clara, CA, USA). Genotypes of each SNP were obtained using the Dynamic Model Mapping Algorithm of the GeneChip Genotyping Analysis Software (GTYPE) 4.1 (Affymetrix). Genotyping coverage was defined as the percentage of SNPs which were successfully assigned a genotype. As such, the SNPs given a 'no call' assignment would contribute to reduced genotyping coverage. Genotyping accuracy was defined as the percentage of SNPs assigned a genotype that was equivalent to the genotype assigned to purified gDNA from the same cell line. Allele dropout (ADO) was defined as the number of SNPs that were assigned a homozygous genotype, despite being assigned a heterozygous genotype in the purified gDNA profiles from the same cell line.

Single-cell WGA CN accuracy

The same data used to evaluate genotyping accuracy above were also evaluated for CN accuracy by using the Copy Number Analysis Tool (CNAT) 4.0.1 (Affymetrix). The CN assignments of each sample were compared with those of the purified gDNA from the same cell line and to the known karyotype of each cell line as reported by the Coriell cell repository. Results were evaluated for accuracy at three levels of analysis; each individual SNP, each individual chromosome and each individual cell's 23 chromosome molecular karyotype. The overall CN assignment

for a single chromosome was determined based on the SNP CN that represented the majority of the assignments within that chromosome (Treff et al., 2010a). Diagnostic accuracy was defined as the percentage of single cells given the correct whole chromosome specific gain, loss or euploid assignments.

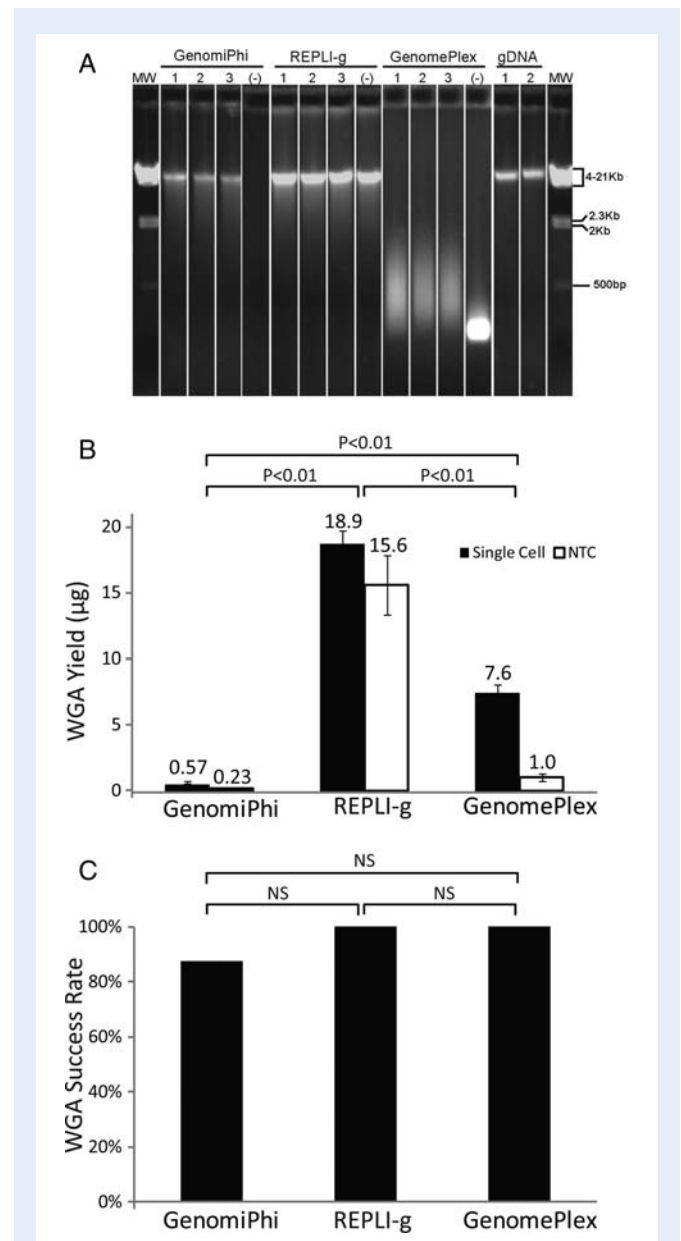


Figure 1 Reliability of single-cell WGA. **(A)** Gel electrophoresis of purified reaction products of three representative samples and one no template control (-) from each of three single-cell WGA methods (GenomiPhi, REPLI-g and GenomePlex). Representative purified gDNA and molecular weight markers (MW) are included for size references. **(B)** The mean yield of amplification (\pm SEM) of 40 single cells (black bars) or 4 no template controls (NTC; white bars) from each of three single-cell WGA methods. **(C)** The rates of successful amplification of 40 single cells from each of three single-cell WGA methods.

Statistics and data repository

A Student's *t*-test was used to evaluate significance; α was set at 0.05. Variation was reported as ± 1 standard error of the mean (SEM). The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE24690 (<http://www.ncbi.nlm.nih.gov/geo>).

Results and Discussion

Both GenomiPhi and REPLI-g methods produced WGA DNA that was equivalent in molecular weight to that of the gDNA (Fig. 1A). However, a similar-sized DNA smear was detected from the no template controls amplified with REPLI-g. As a result, gel electrophoresis

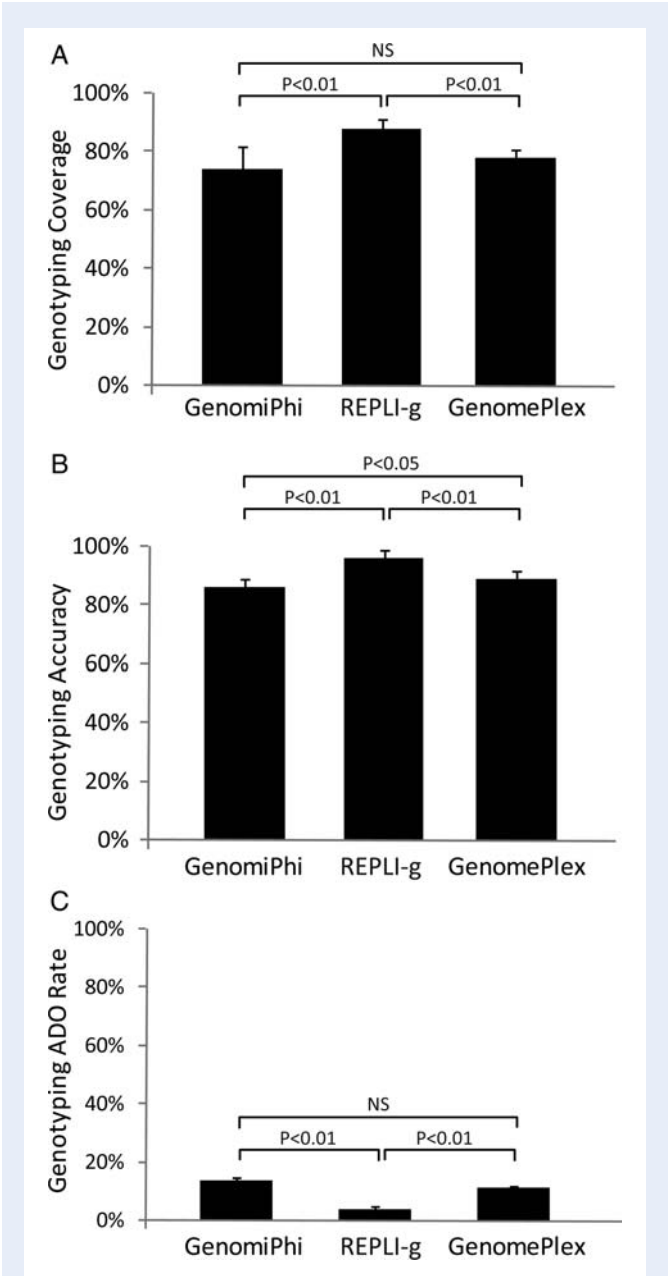


Figure 2 Genotype fidelity of single-cell WGA. **(A)** The percentage of SNPs evaluated that were successfully assigned a genotype (genotyping coverage) for each of three single-cell WGA methods. **(B)** The percentage of SNPs assigned a genotype identical to the purified gDNA assignments (genotyping accuracy) for each of three single-cell WGA methods. **(C)** The percentage of SNPs assigned a homozygous genotype in the single cells but also assigned a heterozygous genotype in the purified gDNA samples (genotyping ADO rate) for each of three single-cell WGA methods.

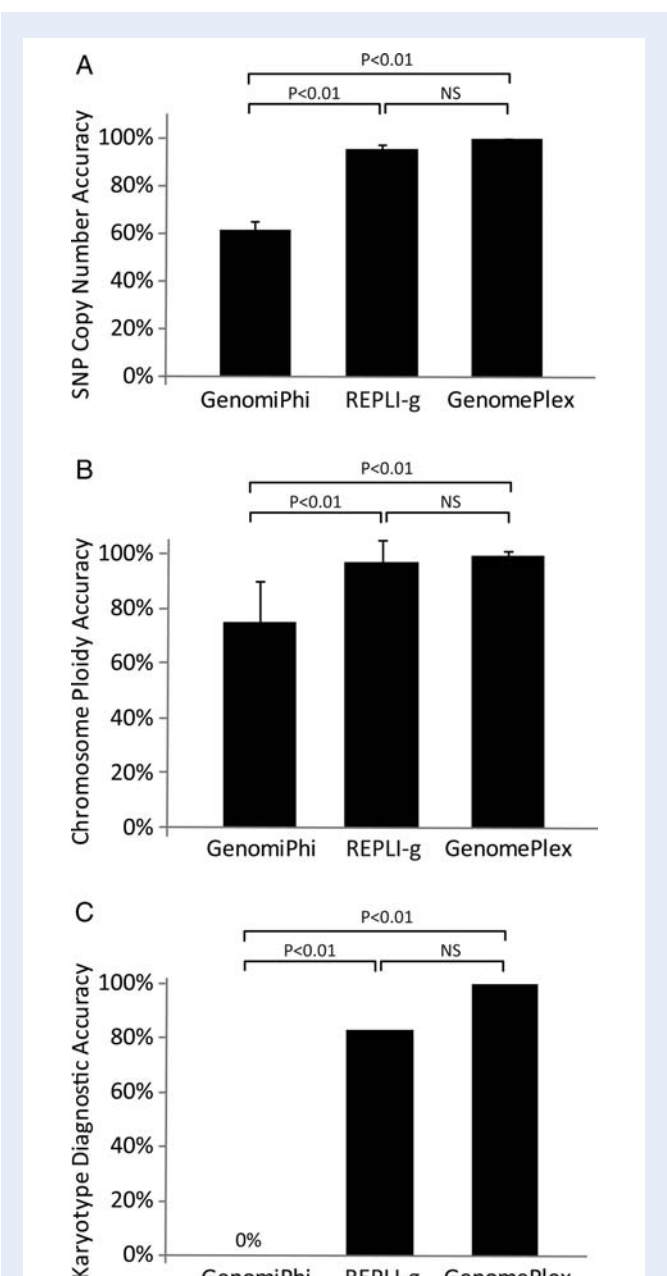


Figure 3 CN assignment accuracy of single-cell WGA. **(A)** The percentage of SNPs evaluated that were assigned the expected CN (SNP CN accuracy) for each of three single-cell WGA methods. **(B)** The percentage of chromosomes evaluated that were assigned the expected CN (chromosome ploidy accuracy) for each of three single-cell WGA methods. **(C)** The percentage of cells that were assigned the expected chromosome loss, gain or euploidy (karyotype diagnostic accuracy) for each of three single-cell WGA methods.

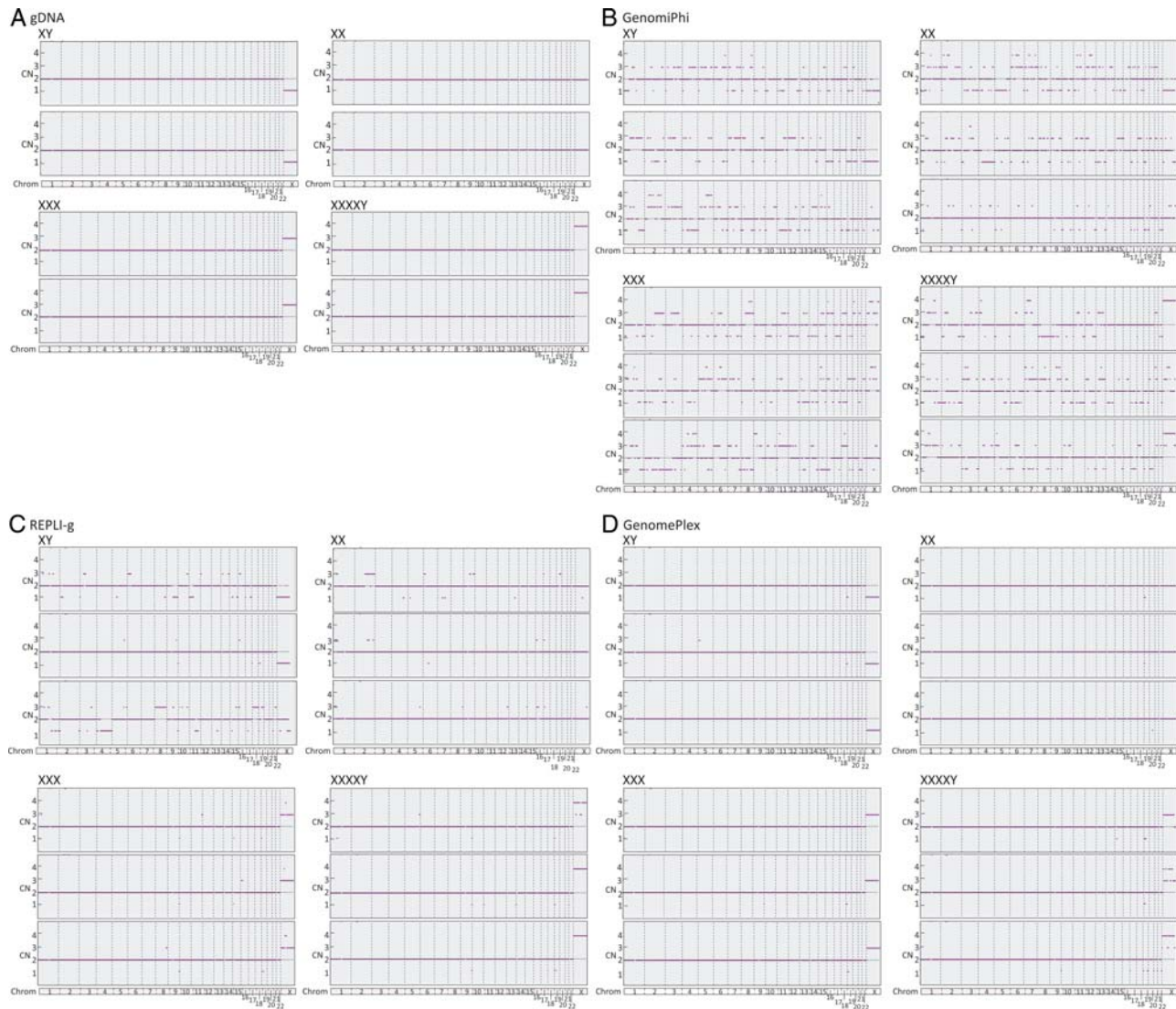


Figure 4 SNP microarray-based CN graphs of (A) purified gDNA, or single cells amplified with (B) GenomiPhi, (C) REPLI-g or (D) GenomePlex protocols. Each panel includes analyses of each of four cell lines possessing one to four X chromosome copies.

of REPLI-g WGA DNA alone was insufficient to determine whether amplification was successful. GenomPlex WGA DNA product size ranged from 100 to 1000 bp (Fig. 1A). The average WGA DNA yield from the GenomiPhi protocol was $0.57 \pm 0.1 \mu\text{g}$ and significantly less ($P < 0.01$) than the $7.63 \pm 0.4 \mu\text{g}$ from GenomePlex or the $18.93 \pm 0.8 \mu\text{g}$ from REPLI-g (Fig. 1B). Similar quantities of DNA were detected from the no template controls amplified using the REPLI-g protocol ($15.62 \pm 2.3 \mu\text{g}$). As a result, DNA quantification by UV spectroscopy of REPLI-g WGA DNA was also insufficient to determine whether specific amplification was successful. This is consistent with previous studies which have found non-specific primer-directed DNA amplification with no template control MDA reactions (Lage et al., 2003; Brukner et al., 2005). Eighty-eight per cent (35/40) of the single cells successfully amplified with the GenomiPhi method by yielding $>250 \text{ ng}$ of WGA DNA. REPLI-g and GenomePlex methods yielded $>250 \text{ ng}$ WGA DNA from 100% of the single cells (Fig. 1C).

No significant difference in reliability of obtaining sufficient quantities of DNA for microarray analysis was observed between the three methods.

Single-cell WGA DNA provided an average of 74% genotyping coverage with the GenomiPhi protocol and 78% with GenomePlex, which were both significantly lower than the 88% obtained with REPLI-g (Fig. 2A). Single-cell WGA DNA genotypes provided an average of 86% accuracy with the GenomiPhi protocol, which was significantly less than the 89% accuracy obtained with GenomePlex (Fig. 2B). Both the GenomiPhi and GenomePlex protocols' genotyping accuracy was significantly lower than the 96% obtained with REPLI-g (Fig. 2B). There was an average ADO rate of 14% using GenomiPhi and 11% using GenomePlex, both of which were significantly higher than the 4% obtained using REPLI-g (Fig. 2C). These results are applicable to performance of methods that require accurate genotyping and qualitative analysis of aneuploidy, such as those described by Johnson

et al. (2010a) and Handyside *et al.* (2010), or in situations where one might consider using WGA DNA to genotype-specific genes of interest (i.e. for single gene disorder screening).

Similarity of single-cell CN assignments to assignments made on gDNA and as expected from the conventional karyotype data for each cell line were evaluated at three levels. For individual SNPs, 62% similarity was obtained using the GenomiPhi protocol, which was significantly less than the 95% similarity obtained using REPLI-g or the 99% similarity obtained using GenomePlex (Fig. 3A). For individual chromosomes, 75% similarity was obtained using the GenomiPhi protocol, which was significantly less than the 97% similarity obtained using REPLI-g or the 99% similarity obtained using GenomePlex (Fig. 3B). For single-cell molecular karyotyping diagnosis, 0% accuracy was obtained using the GenomiPhi protocol, which was significantly less than the 83% similarity obtained using REPLI-g or the 100% similarity obtained using GenomePlex (Fig. 3C). A comprehensive view of the gDNA and single-cell CN assignments is also displayed in Fig. 4 and reflects the levels of accuracy reported above. These results are of particular importance to the performance of methods that require accurate quantitative analysis of CN such as those reported by Le Caignec *et al.* (2006) and Vanneste *et al.* (2009), which used GenomiPhi technology, and Fiegler *et al.* (2007), Treff *et al.* (2010a) and Gutierrez-Mateo *et al.* (2010), which used GenomePlex technology.

The duration of amplification is also important when considering the application of single-cell WGA technology to clinical PGD. With WGA only one step is necessary to generate a diagnosis for the amplified sample, which also involves downstream microarray processing and analysis. For example, the most typical PGD application requires the completion of single-cell analysis within 24 h of initiating the procedure in order to avoid embryo cryopreservation. Therefore, although the REPLI-g protocol may be suitable for single-cell applications that do not have time constraints, the 16 h turnaround time may not allow for its routine use in PGD for aneuploidy screening. A more rapid turnaround time with isothermal MDA was represented in this study by the GenomiPhi protocol. Unfortunately, this shortened MDA protocol performed with the least reliability, fidelity and accuracy of all methods tested. In contrast, the GenomePlex protocol provided a more rapid turnaround time (4 h) which could be suitable for application to PGD and produced the highest CN assignment accuracy of all methods tested. Therefore, for applications requiring accurate and rapid CN analysis, such as PGD for aneuploidy screening, the GenomePlex protocol may be more appropriate than REPLI-g or GenomiPhi MDA-based protocols. However, for those applications requiring accurate genotyping analysis without time constraints, the REPLI-g protocol may be more appropriate than the GenomePlex or GenomiPhi protocols.

In summary, this study represents the first direct comparison of commercially available single-cell WGA method performance, a necessary step in all 24 chromosome aneuploidy screening technologies. Clinically relevant measurements of reliability, fidelity and accuracy were evaluated for each method. In general, a longer MDA protocol was better for genotyping accuracy than PCR, and PCR was better and faster than MDA for CN accuracy. Clinicians and laboratory directors should consider these and other critical pieces of evidence (presented in Table 1 and reviewed in Scott and Treff, 2010) when evaluating new technologies that intend to

predict the chromosomal status and reproductive potential of human embryos.

Authors' roles

N.R.T. and R.T.S. designed the study, N.R.T., J.S. and L.E.N. wrote the manuscript, and J.S., X.T. and L.E.N. performed the experiments and prepared the microarray data for publication.

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